

UNITED STATES CONTINUING UTILITY PATENT APPLICATION
under 37 C.F.R. § 1.53(b)

04/26/00
C639
PTO

Page 1

Atty. Docket No. 01107.00004

JC571 U.S. PTO
09/558149
04/26/00

Assistant Commissioner of Patents
Box Patent Applications
Washington, D.C. 20231

Enclosed herewith is a continuing patent application and the following papers:

First Named Inventor (or application identifier): Nicholas Nicolaides

Title of Invention: A METHOD FOR GENERATING HYPERMUTABLE ORGANISMS

- Continuation
- Divisional
- Continuation-in-Part

of prior application No. 09/059,461, filed April 14, 1998

1. Specification 43 pages (including specification, claims, abstract) / 59 claims (5 independent)
2. Declaration/Power of Attorney:
 - Copy from Prior Application (for continuation or divisional application)
 - Newly Executed Declaration (for CIP application)
 - Deferred under 37 C.F.R. § 1.53(f)
 - Deletion of Inventor(s) - Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b)
 - Incorporation by Reference - The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein
3. 6 Distinct sheets of Formal Informal Drawings
4. Preliminary Amendment
5. Information Disclosure Statement
 - Form 1449
 - A copy of each cited prior art reference
6. Assignment
 - Assignment with Cover Sheet attached
 - Assignment filed in prior application. Application assigned to: **The Johns Hopkins University**
7. Priority is hereby claimed under 35 U.S.C. § 119 based upon the following application(s):

UNITED STATES CONTINUING UTILITY PATENT APPLICATION
 under 37 C.F.R. § 1.53(b)

Page 2

Atty. Docket No. 01107.00004

Country	Application Number	Date of Filing (day, month, year)

8. Priority document(s)

9. Small Entity Statement

- Small Entity Statement was filed in prior application, Small Entity Status is still proper and desired
- is attached
- is no longer claimed

10. Microfiche Computer Program (Appendix)

11. Nucleotide and/or Amino Acid Sequence Submission

- Computer Readable Copy
- Paper Copy (identical to computer copy)
- Statement verifying identity of above copies

12. Calculation of Fees:

FEES FOR	EXCESS CLAIMS	FEE	AMOUNT DUE
Basic Filing Fee (37 C.F.R. § 1.16(a))			\$690.00
Total Claims in Excess of 20 (37 C.F.R. § 1.16(c))	0	18.00	\$0.00
Independent Claims in Excess of 3 (37 C.F.R. § 1.16(b))	0	78.00	\$0.00
Multiple Dependent Claims (37 C.F.R. § 1.16(d))	0	260.00	\$0.00
Subtotal - Filing Fee Due			\$690.00
		MULTIPLY BY	
Reduction by 50%, if Small Entity (37 C.F.R. §§ 1.9, 1.27, 1.28)	0.50		\$345.00
TOTAL FILING FEE DUE			\$345.00
Assignment Recordation Fee (if applicable) (37 C.F.R. § 1.21(h))	0	40.00	\$0.00
GRAND TOTAL DUE			\$345.00

13. PAYMENT is:

included in the amount of the GRAND TOTAL by our enclosed check. A general authorization under 37 C.F.R. § 1.25(b), second sentence, is hereby given to credit or debit our Deposit Account No. 19-0733 for the instant filing and for any other fees during the pendency of this application under 37 C.F.R. §§ 1.16, 1.17 and 1.18

not included, but deferred under 37 C.F.R. § 1.53(f).

UNITED STATES CONTINUING UTILITY PATENT APPLICATION
under 37 C.F.R. § 1.53(b)

Page 3

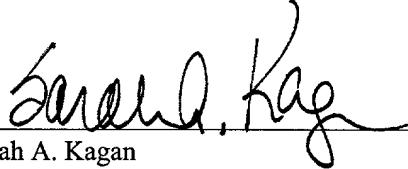
Atty. Docket No. 01107.00004

14. All correspondence for the attached application should be directed to:

Banner & Witcoff, Ltd.
1001 G Street, N.W.
Washington, D. C. 20001-4597
Telephone: (202) 508-9100
Facsimile: (202) 508-9299

15. Other: Please make a computer readable form for examination of this application from the computer readable form submitted in parent application Serial No. 09/059,461. The sequence content of the computer readable form and the paper copy in the application are believed to be identical.

Date: April 25, 2000

By: 
Sarah A. Kagan
Reg. No. 32,141

SAK/ama

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)
)
)
Nicholas Nicolaides et al.)
)
)
Serial No. T/B/A)
)
)
Filed: April 25, 2000) Atty. Dkt. No. 01107.00004

For: **METHOD FOR GENERATING HYPERMUTABLE ORGANISMS**

Divisional Application based on Serial No. 09/059,461, filed April 14, 1998

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents & Trademarks
Washington, D.C. 20231

Sir:

Please enter the following amendments before examining the continuation application which is filed concurrently herewith.

IN THE SPECIFICATION:

Page 1, between lines 2 and 3 insert:

--This application is a division of U.S. Serial No. 09/059,461, filed April 14, 1998, allowed.--

IN THE CLAIMS

Please delete claims 1-12, 15-17, 21-28, 30-51, and 54-57.

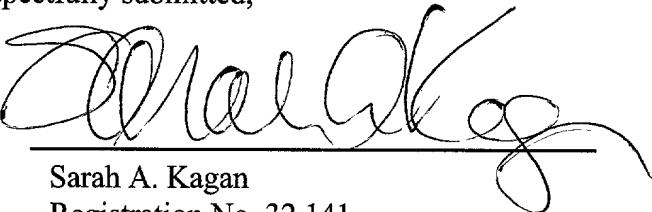
REMARKS

The present application is directed to claims 13, 14, 18-20, 29, 52, 53, 58 and 59.

It is believed that no fee is required to make this a complete and timely filing. However, if a fee is required, the Commissioner is authorized to charge Deposit Account No. 19-0733.

Respectfully submitted,

By:


Sarah A. Kagan
Registration No. 32,141

Date: April 25, 2000

Banner & Witcoff, Ltd.
1001 G Street, N.W., Eleventh Floor
Washington, D.C. 20001-4597
(202) 508-9100

SAK/ama

A METHOD FOR GENERATING HYPERMUTABLE ORGANISMS

This invention was made using a U.S. government grant from the NIH (CA43460). Therefore, the U.S. government retains certain rights to the invention.

5

TECHNICAL FIELD OF THE INVENTION

The invention is related to the area of mismatch repair genes. In particular it is related to the field of mutagenesis.

BACKGROUND OF THE INVENTION

Within the past four years, the genetic cause of the Hereditary Nonpolyposis Colorectal Cancer Syndrome (HNPCC), also known as Lynch syndrome II, has been ascertained for the majority of kindreds affected with the disease (13). The molecular basis of HNPCC involves genetic instability resulting from defective mismatch repair (MMR). To date, six genes have been identified in humans that encode for proteins and appear to participate in the MMR process, including the *mutS* homologs *GTBP*, *hMSH2*, and *hMSH3* and the *mutL* homologs *hMLH1*, *hPMS1*, and *hPMS2* (2,7,11,17,20,21,22, 24). Germline mutations in four of these genes (*hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2*) have been identified in HNPCC

kindreds (2,11,13,17,24). Though the mutator defect that arises from the MMR deficiency can affect any DNA sequence, microsatellite sequences are particularly sensitive to MMR abnormalities (14). Microsatellite instability is therefore a useful indicator of defective MMR. In addition to its occurrence in virtually all tumors arising in HNPCC patients, Microsatellite instability is found in a small fraction of sporadic tumors with distinctive molecular and phenotypic properties (27).

HNPCC is inherited in an autosomal dominant fashion, so that the normal cells of affected family members contain one mutant allele of the relevant MMR gene (inherited from an affected parent) and one wild-type allele (inherited from the unaffected parent). During the early stages of tumor development, however, the wild-type allele is inactivated through a somatic mutation, leaving the cell with no functional MMR gene and resulting in a profound defect in MMR activity. Because a somatic mutation in addition to a germ-line mutation is required to generate defective MMR in the tumor cells, this mechanism is generally referred to as one involving "two hits," analogous to the biallelic inactivation of tumor suppressor genes that initiate other hereditary cancers (11,13,25). In line with this two-hit mechanism, the non-neoplastic cells of HNPCC patients generally retain near normal levels of MMR activity due to the presence of the wild-type allele.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for rendering cells hypermutable.

It is another object of the present invention to provide genetically altered cell lines.

It is yet another object of the present invention to provide a method to produce transgenic animals that are hypermutable.

It is also an object of the present invention to provide genetically altered transgenic animals.

It is a further object of the invention to provide a method of mutating a gene of interest in a cell.

Yet another object of the invention is to provide a method of mutating a gene of interest in an animal.

5

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment of the invention, a method for making a hypermutable cell is provided. A polynucleotide encoding a dominant negative allele of a mismatch repair gene is introduced into a cell. The cell becomes hypermutable as a result of the introduction of the gene.

10

In another embodiment of the invention, an isolated hypermutable cell is provided. The cell comprises a dominant negative allele of a mismatch repair gene.

15

In another embodiment of the invention, a hypermutable transgenic animal is provided. The animal comprises a dominant negative allele of a mismatch repair gene.

20

In another embodiment of the invention, a method is provided for introducing a mutation into a gene of interest. A polynucleotide encoding a dominant negative allele of a mismatch repair gene is introduced into a cell. The cell becomes hypermutable as a result of the introduction of the gene. The cell further comprises a gene of interest. The cell is grown. The cell is tested to determine whether the gene of interest harbors a mutation.

25

In another embodiment of the invention, a method is provided for generating a mutation in a gene of interest. A transgenic animal comprising a polynucleotide encoding a dominant negative allele of a mismatch repair gene is grown. The animal comprises a gene of interest. The animal is tested to determine whether the gene of interest harbors a mutation.

30

These and other embodiments of the invention provide the art with methods that can generate enhanced mutability in cells and animals as well as providing cells and animals harboring potentially useful mutations.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Diagrams of *PMS2* expression vectors (Fig. 1A) and pCAR reporters (Fig. 1B).

5

Figure 2. SH cells co-transfected with pCAR reporters and *PMS2* expression vectors after 17 days of drug selection. (Fig. 2A) Western blots of lysates from untransfected SH cells (lane 1) or SH cells transfected with *PMS2-NOT* (lane 2) or *PMS2-WT* (lane 3). The arrow indicates the 110 kD protein expected for hPMS2. (Fig. 2B) Western blots of lysates from untransfected SH cells (lane 1) or SH cells transfected with *PMS2-NOT* (lane 2) or *PMS2-134* (lane 3). The arrow indicates the 14 kD protein expected for hPMS-134. Both A and B were probed with an antibody generated against the N-terminus of hPMS2. The upper polypeptides in A and the lower polypeptides in B represent cross-reactive hamster proteins. (Fig. 2C) β -galactosidase activity in lysates derived from SH cells co-transfected with *PMS2-NOT* (lane 1), *PMS2-WT* (lane 2), or *PMS2-134* (lane 3) plus reporter plasmid. Relative β -galactosidase activities are defined as the ratio of β -galactosidase activity in cells transfected with pCAR-OF compared to that in cells transfected with pCAR-IF; this normalization controlled for transfection efficiency and controlled for β -galactosidase activity in the cells expressing the various *PMS2* effector genes.

25

Figure 3. *In situ* β -galactosidase activity of pooled clones of SH cells stably transduced with the *PMS2-NOT* (Fig. 3A), *PMS2-WT* (Fig. 3B), or *PMS2-134* (Fig. 3C) expression vectors, then re-transfected with pCAR-OF reporter. After 17 days of drug selection, the colonies were pooled, cultured, and stained for β -galactosidase activity. A pooled culture of of *PMS2-134* transduced SH cells expressing β -galactosidase from pCAR-OF

DISCOVER YOUR PATENT

is visible in Fig. 3C. The level of expression is lower, as expected, than in SH cells transfected with the pCAR-IF reporter plasmid, shown as a positive control in Fig. 3D. Each of the fields illustrated is representative of that found in triplicate experiments.

5

Figure 4. Protein expression and β -galactosidase activity in stably transduced SH clones. (Fig. 4A) Western blots of lysates from clones stably transduced with PMS2-NOT (lanes 1-3) or PMS2-WT (lanes 4-6). (Fig. 4B) Western blots of lysates from clones stably transduced with PMS2-NOT (lanes 1-3) or PMS2-134 (lanes 4-6). (The arrows indicate the polypeptide of the appropriate molecular weight. The upper (Fig. 4A) and lower (Fig. 4B) molecular weight polypeptides are nonspecific proteins. (Fig. 4C) The clones expressing PMS2-NOT (lane 1A-3A), PMS2-WT (lanes 1B-3B), or PMS2-134 (lanes 1C-3C) were transduced with pCAR-OF or pCAR-IF reporter plasmids and multiple subclones selected in hygromycin plus geneticin were harvested 17 days later and assayed for β -galactosidase activity. Relative β -galactosidase activities are defined as the ratio of β -galactosidase activity in cells transduced with pCAR-OF compared to that in cells transduced with pCAR-IF.

10

Figure 5. Immunoprecipitation of *in vitro* translated hPMS2 and hMLH1 proteins. (Fig. 5A) Labelled (indicated by an asterisk) or unlabelled proteins were incubated with an antibody to the C-terminus of hPMS2 in lanes 1-3 and to hMLH1 in lanes 4-6. Lane 7 contains a nonprogrammed reticulocyte lysate. The PMS-135 contains codons 135-862 of hPMS2. The major translation products of hPMS2 and hMLH1 are indicated. (Fig. 5B) Labelled hPMS-134 (containing codons 1-134 of hPMS2) was incubated in the presence or absence of unlabelled hMLH1 plus an antibody to hMLH1 (lanes 1 and 2, respectively). Lane 3 contains lysate from a nonprogrammed reticulolysate. (Fig. 5C) Labelled proteins were incubated with an antibody to the N-terminus of hPMS2. Lane 6 contains a nonprogrammed

15

20

25

30

RECEIVED JULY 26, 1989

reticulocyte lysate. In both Fig. 5A and Fig. 5B, autoradiographs of immunoprecipitated products are shown.

Figure 6. Complementation of MMR activity in transduced SH cells.

5 Lysates from pooled clones stably transduced with *PMS2-NOT*, *PMS2-WT*, or *PMS2-134* were complemented with purified *MutS α* or *MutL α* MMR components using the 5'G/T heteroduplex substrate. The values are presented as the percentage of repair activity in each case compared to that in lysates complemented with both purified *MutL α* and *MutS α* components to normalize for repair efficiency in the different lysate backgrounds. The values shown represent the average of at least three different determinations.

DETAILED DESCRIPTION OF THE INVENTION

10 The inventors have discovered a method for developing hypermutable cells and animals by taking advantage of newly discovered alleles of human mismatch repair genes. Dominant negative alleles of such genes, when introduced into cells or transgenic animals, increase the rate of spontaneous mutations by reducing the effectiveness of DNA repair and thereby render the cells or animals hypermutable. Hypermutable cells or 15 animals can then be utilized to develop new mutations in a gene of interest.

20 The process of mismatch repair, also called mismatch proofreading, is carried out by protein complexes in cells ranging from bacteria to mammalian cells. A mismatch repair gene is a gene that encodes one of the proteins of such a mismatch repair complex. Although not wanting to be bound by any particular theory of mechanism of action, a mismatch repair 25 complex is believed to detect distortions of the DNA helix resulting from non-complementary pairing of nucleotide bases. The non-complementary base on the newer DNA strand is excised, and the excised base is replaced with the appropriate base which is complementary to the older DNA strand. 30 In this way, cells eliminate many mutations which occur as a result of mistakes in DNA replication.

SEARCHED
INDEXED
COPIED
FILED

5

10

15

20

25

30

Dominant negative alleles cause a mismatch repair defective phenotype even in the presence of a wild-type allele in the same cell. An example of a dominant negative allele of a mismatch repair gene is the human gene *hPMS2-134*, which carries a truncation mutation at codon 134. The mutation causes the product of this gene to abnormally terminate at the position of the 134th amino acid, resulting in a shortened polypeptide containing the N-terminal 133 amino acids. Such a mutation causes an increase in the rate of mutations which accumulate in cells after DNA replication. Expression of a dominant negative allele of a mismatch repair gene results in impairment of mismatch repair activity, even in the presence of the wild-type allele. Any allele which produces such effect can be used in this invention.

Dominant negative alleles of a mismatch repair gene can be obtained from the cells of humans, animals, yeast, bacteria, or other organisms. Such alleles can be identified by screening cells for defective mismatch repair activity. The cells may be mutagenized or not. Cells from animals or humans with cancer can be screened for defective mismatch repair. Cells from colon cancer patients may be particularly useful. Genomic DNA, cDNA, or mRNA from any cell encoding a mismatch repair protein can be analyzed for variations from the wild type sequence. Dominant negative alleles of a mismatch repair gene can also be created artificially, for example, by producing variants of the *hPMS2-134* allele or other mismatch repair genes. Various techniques of site-directed mutagenesis can be used. The suitability of such alleles, whether natural or artificial, for use in generating hypermutable cells or animals can be evaluated by testing the mismatch repair activity caused by the allele in the presence of one or more wild-type alleles, to determine if it is a dominant negative allele.

A cell or an animal into which a dominant negative allele of a mismatch repair gene has been introduced will become hypermutable. This means that the spontaneous mutation rate of such cells or animals is elevated compared to cells or animals without such alleles. The degree of elevation

0 9 5 5 8 1 1 0 0 * 0 7 7 6 0 0

of the spontaneous mutation rate can be at least 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, or 1000-fold that of the normal cell or animal.

According to one aspect of the invention, a polynucleotide encoding
5 a dominant negative form of a mismatch repair protein is introduced into a cell or a transgenic animal. The gene can be any dominant negative allele encoding a protein which is part of a mismatch repair complex, for example, *PMS2*, *PMS1*, *MLH1*, or *MSH2*. The dominant negative allele can be naturally occurring or made in the laboratory. The polynucleotide can be in
10 the form of genomic DNA, cDNA, RNA, or a chemically synthesized polynucleotide. The polynucleotide can be introduced into the cell by transfection.

Transfection is any process whereby a polynucleotide is introduced
15 into a cell. The process of transfection can be carried out in a living animal, e.g., using a vector for gene therapy, or it can be carried out *in vitro*, e.g., using a suspension of one or more isolated cells in culture. The cell can be any type of eukaryotic cell, including, for example, cells isolated from humans or other primates, mammals or other vertebrates, invertebrates, and single celled organisms such as protozoa or yeast.

20 In general, transfection will be carried out using a suspension of cells, or a single cell, but other methods can also be applied as long as a sufficient fraction of the treated cells or tissue incorporates the polynucleotide so as to allow transfected cells to be grown and utilized. The protein product of the polynucleotide may be transiently or stably expressed in the cell. Techniques for transfection are well known. Available
25 techniques for introducing polynucleotides include but are not limited to electroporation, transduction, cell fusion, the use of calcium chloride, and packaging of the polynucleotide together with lipid for fusion with the cells of interest. Once a cell has been transfected with the mismatch repair gene, the cell can be grown and reproduced in culture. If the transfection is stable,
30

such that the gene is expressed at a consistent level for many cell generations, then a cell line results.

An isolated cell is a cell obtained from a tissue of humans or animals by mechanically separating out individual cells and transferring them to a suitable cell culture medium, either with or without pretreatment of the tissue with enzymes, e.g., collagenase or trypsin. Such isolated cells are typically cultured in the absence of other types of cells. Cells selected for the introduction of a dominant negative allele of a mismatch repair gene may be derived from a eukaryotic organism in the form of a primary cell culture or an immortalized cell line, or may be derived from suspensions of single-celled organisms.

A polynucleotide encoding a dominant negative form of a mismatch repair protein can be introduced into the genome of an animal by producing a transgenic animal. The animal can be any species for which suitable techniques are available to produce transgenic animals. For example, transgenic animals can be prepared from domestic livestock, e.g., cows, pigs, sheep, goats, horses, etc.; from animals used for the production of recombinant proteins, e.g., cows, pigs, or goats that express a recombinant protein in their milk; or experimental animals for research or product testing, e.g., mice, rats, hamsters, guinea pigs, rabbits, etc.

Any method for making transgenic animals known in the art can be used. According to one process of producing a transgenic animal, the polynucleotide is injected into a fertilized egg of the animal and the injected egg is placed into a pseudo-pregnant female. The egg develops into a mature animal in which the polynucleotide is incorporated and expressed. The fertilized egg is produced *in vitro* from the egg and sperm of donor animals of the same species as the pseudo-pregnant female, who is prepared by hormone treatments to receive the fertilized egg and become pregnant. An alternative method for producing transgenic animals involves introducing the polynucleotide into embryonic cells by injection or transfection and reintroducing the embryonic cells into the developing embryo. With this

method, however, if the polynucleotide is not incorporated into germline cells, the gene will not be passed on to the progeny. Therefore, a transgenic animal produced by this method must be evaluated to determine whether the gene is incorporated into germ cells of the animal. Once transgenic animals are produced, they can be grown to reproductive age, when they can be mated to produce and maintain a colony of transgenic animals.

Once a transfected cell line or a colony of transgenic animals has been produced, it can be used to generate new mutations in one or more gene(s) of interest. A gene of interest can be any gene naturally possessed by the cell line or transgenic animal or introduced into the cell line or transgenic animal. An advantage of using such cells or animals to induce mutations is that the cell or animal need not be exposed to mutagenic chemicals or radiation, which may have secondary harmful effects, both on the object of the exposure and on the workers.

Mutations can be detected by analyzing for alterations in the genotype of the cells or animals, for example by examining the sequence of genomic DNA, cDNA, messenger RNA, or amino acids associated with the gene of interest. Mutations can also be detected by screening the phenotype of the gene. A mutant phenotype can be detected by identifying alterations in electrophoretic mobility, spectroscopic properties, or other physical or structural characteristics of a protein encoded by a mutant gene. One can also screen for altered function of the protein *in situ*, in isolated form, or in model systems. One can screen for alteration of any property of the cell or animal associated with the function of the gene of interest.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1: *hPMS2-134* Encodes a Dominant Negative Mismatch Repair Protein.

A profound defect in MMR was found in the normal cells of two HNPCC patients. That this defect was operative *in vivo* was demonstrated by the widespread presence of microsatellite instability in non-neoplastic cells of such patients. One of the two patients had a germ-line truncating mutation of the *hPMS2* gene at codon 134 (the *hPMS2-134* mutation), while the other patient had a small germ-line deletion within the *hMLH1* gene (26). These data thus contradicted the two-hit model generally believed to explain the biochemical and biological features of HNPCC patients. The basis for this MMR deficiency in the normal cells of these patients was unclear, and several potential explanations were offered. For example, it was possible that the second allele of the relevant MMR gene was inactivated in the germ-line of these patients through an undiscovered mechanism, or that unknown mutations of other genes involved in the MMR process were present that cooperated with the known germ-line mutation. It is clear from knock-out experiments in mice that MMR-deficiency is compatible with normal growth and development, supporting these possibilities (1,3,6). Alternatively, it was possible that the mutant alleles exerted a dominant negative effect, resulting in MMR deficiency even in the presence of the wild-type allele of the corresponding MMR gene and all other genes involved in the MMR process. To distinguish between these possibilities, we expressed the truncated polypeptide encoded by the *hPMS2-134* mutation in an MMR proficient cell line and analyzed its affect on the cell's MMR activity. The results showed that this mutant could indeed exert a dominant negative effect, resulting in biochemical and genetic manifestations of MMR deficiency.

The MMR proficient Syrian hamster TK-ts13 cell line (hereafter called SH cells) was cotransfected with various *hPMS2* expression plasmids plus reporter constructs for assessing MMR activity. The *hPMS2* expression plasmids contained the normal *hPMS2* gene product or the

truncated *hPMS2* gene identified in the patient described above (*PMS2-WT* and *PMS2-134*, respectively, Fig. 1A). An "empty" vector devoid of *hPMS2* sequences (*PMS2-NOT*, Fig. 1A) served as an additional control. The reporter construct pCAR-OF (out of frame) contained a hygromycin
5 resistance gene plus a β -galactosidase gene containing a 29 bp out-of-frame poly-CA tract at the 5' end of its coding region. The reporter construct pCAR-IF (in frame) was identical except that the poly-CA tract was 27 bp and therefore did not disrupt the β -galactosidase reading frame (Fig. 1B).
10 The pCAR-OF reporter would not generate β -galactosidase activity unless a frame-restoring mutation (*i.e.*, insertion or deletion) arose following transfection.

Three different transfection schemes were used to evaluate the effects of the *PMS2-134* mutation on SH cells. In the first scheme, the expression vectors plus the reporters were co-transfected together.
15 Pools containing greater than 100 clones were generated following selection with hygromycin for 17 days and harvested for Western blot and β -galactosidase assays. SH cells transduced with *PMS2-WT* and *PMS2-134* synthesized polypeptides of the expected size, as assessed with anti-hPMS2 antibodies on Western blots (Fig. 2A and 2B). As expected, virtually no β -galactosidase activity was observed in SH
20 cells transfected with the pCAR-OF reporter plus *PMS2-NOT* (Fig. 2C). However, SH cells transfected with *PMS2-134* expressed considerable β -galactosidase activity, significantly more than those transfected with *PMS2-WT* (Fig. 2C). These results suggested that
25 the truncated polypeptide encoded by the *PMS2-134* construct perturbs the endogenous MMR machinery, resulting in deletions or insertions that restored the reading frame. The exact nature of these presumed deletions or insertions could not be assessed, as multiple copies of the reporter constructs were transduced under our
30 conditions, and the wild type β -galactosidase sequence was in great

DECODED BY DR. J. D. COOPER

excess over the expected mutants, precluding their demonstration by direct sequencing.

In the second scheme, SH cells were co-transfected with each of the PMS2 expression vectors plus the hygromycin-resistance plasmid pLHL4. Hygromycin resistant cultures containing greater than 100 clones were pooled and expanded. These cultures were then co-transfected with pCAR-IF or pCAR-OF reporters plus a separate plasmid allowing geneticin selection. Two weeks later, the pooled cells, each containing more than 100 colonies resistant to both hygromycin and geneticin, were stained with X-gal to assess β -galactosidase activity. As shown in Figure 3, the cultures transfected with *PMS2-134* (panel C) contained many blue cells, while virtually no cells were blue in the cultures transfected with *PMS2-NOT* or *PMS2-WT* (panels A and B, respectively). In each case, transfection efficiency was controlled by parallel transfections using pCAR-IF which also served as a control for β -galactosidase activity of cells expressing the various PMS2 effector genes, which resulted in similar β -galactosidase expression levels in all cases (example in Fig. 3D). Increases in β -galactosidase activity after *PMS2-134* transfection compared to *PMS2-WT* transfection were also observed when a similar experimental protocol was applied to the MMR-proficient human embryonic kidney cell line 293. These cells were cotransfected with the pCAR-OF plus the various PMS2 effector plasmids and selected for 17 days in hygromycin. At day 17, colonies were stained with X-gal to assess β -galactosidase activity and scored for β -galactosidase expressing cells. As shown in Table 1, only those cells expressing the PMS2-134 polypeptide expressed a detectable β -galactosidase activity. These data demonstrate a similar dominant negative effect of the hPMS2-134 protein in both rodent and human systems and validate the utility of the rodent system in these studies.

D E S E G R A D E D

In the third scheme, SH cells were transfected with each of the *PMS2* expression vectors as described for the second scheme, but individual clones , rather than pooled clones, were expanded following drug selection. Of twenty clones transfected with *PMS2-WT*, five
5 were shown to express readily detectable levels of full-length PMS2 proteins (examples in Fig. 4A, lanes 4-6). Similar analyses of twenty *PMS2-134* clones revealed four clones which expressed truncated PMS2 polypeptides of the expected size (examples in Figure 4B, lanes 4-6). Three clones expressing full-length or truncated PMS2
10 proteins, as well as three randomly selected clones from *PMS2-NOT* transfected cells (Figure 4A and 4B, lanes 1-3) were chosen for further analysis. The individual clones were tested for β -galactosidase activity following co-transfection with pCAR-OF plus the pNTK plasmid, as described above for the pooled clones. As shown in
15 Figure 4C, each of the three clones (lanes 3A-3C) expressing the truncated hPMS2 polypeptide yielded much higher β -galactosidase activities following transfection with pCAR-OF than did the clones expressing the full-length hPMS2 protein (lanes 2A-2C) or no hPMS2 protein (lanes 1A-1C).

20

Table 1. β -galactosidase expression of 293 clones transfected with pCAR-OF reporter construct plus PMS2 effector plasmids. 293 cells were cotransfected with the pCAR-OF β -galactosidase reporter plasmid plus the *PMS2-NOT*, *-WT*, or *-I34* effector plasmids.

5 Transfected cells were selected in hygromycin for 17 days and stained with x-gal for β -galactosidase activity (blue colored cells). The results below represent the mean +/- standard deviation of triplicate experiments.

10	<u>Sample</u>	<u>Blue colonies</u>	<u>White colonies</u>
15	<i>PMS2-NOT</i>	0 +/- 0	17 +/- 2.7
	<i>PMS2-WT</i>	0 +/- 0	18 +/- 4.0
	<i>PMS2-I34</i>	15 +/- 2.1	6 +/- 2.1

5 Plasmids. The full-length wild-type *hPMS2* cDNA was obtained from a human Hela cDNA library as described (18). An *hPMS2* cDNA containing a termination codon at amino acid 134 was obtained via RT-PCR from the patient in which the mutation was discovered (9). The cDNA fragments were cloned into the BamHI site into the pSG5 vector, which contains an SV40 promoter followed by an SV40 polyadenylation signal (8). The pCAR reporter vectors described in Fig. 1 were constructed as described in ref. 21 and 25.

10 Cell lines and transfection. Syrian Hamster fibroblast Tk-ts13 cells were obtained from ATCC and cultured as described (15). Stably transfected cell lines expressing *hPMS2* were created by cotransfection of the *PMS2* expression vectors and the pLHL4 plasmid encoding the hygromycin resistance gene at a ratio of 3:1 (pCAR:pLHL4) and selected with hygromycin. Stably transfected cell lines containing pCAR reporters were generated by co-transfection of pCAR vectors together with either pNTK plasmid encoding the neomycin resistance plasmid or with pLHL4. All transfections were performed using calcium phosphate as previously described (15).

15 β -galactosidase assay. Seventeen days following transfection with pCAR, β -galactosidase assays were performed using 20 μ g of protein in 45 mM 2-mercaptoethanol, 1mM MgCl₂, 0.1 M NaPO₄ and 0.6 mg/ml Chlorophenol red- β -D-galatopyranoside (CPRG, Boehringer Mannheim). Reactions were incubated for 1 hour, terminated by the addition of 0.5 M Na₂CO₃, and analyzed by spectrophotometry at 576 nm (16). For *in situ* β -galactosidase staining, cells were fixed in 1% glutaraldehyde in PBS and incubated in 0.15 M NaCl, 1 mM MgCl₂, 3.3 mM K₄Fe(CN)₆, 3.3 mM K₃Fe(CN)₆, 0.2% X-Gal for 2 hours at 37°C.

EXAMPLE 2: hPMS2-134 Causes a Defect in MMR Activity

The most likely explanation for the differences in β-galactosidase activity between *PMS2-WT* and *PMS2-134* transfected cells was that the PMS2-134 protein disturbed MMR activity, resulting in a higher frequency of mutation within the pCAR-OF reporter and re-establishing the ORF. To directly test the hypothesis that MMR was altered, we employed a biochemical assay for MMR with the individual clones described in Fig. 4. Nuclear extracts were prepared from the clones and incubated with heteroduplex substrates containing either a /CA\ insertion-deletion or a G/T mismatch under conditions described previously. The /CA\ and G/T heteroduplexes were used to test repair from the 3' and 5' directions, respectively. There was a dramatic difference between the *PMS2-134* expressing clones and the other clones in these assays (Table 2A). While all clones repaired substrates from the 3' direction (/CA\ heteroduplex), cells expressing the PMS2-134 polypeptide had very little 5' repair activity. A similar directional defect in mismatch repair was evident with pooled clones resulting from *PMS2-134* transfection, or when the heteroduplex contained a 2-4 base pair loop, examples of which are shown in Table 2B. A small decrease in MMR activity was observed in the 3' /CA\ *PMS2-WT* repair assays, perhaps a result of interference in the biochemical assays by overexpression of the PMS2 protein. No significant activity was caused by *PMS2-WT* in the *in situ* β-galactosidase assays (Fig. 3; Table1), a result more likely to reflect the *in vivo* condition.

DRAFT DATE: 04/26/2018

Table 2. Mismatch repair activity of nuclear extracts from SH clones (A) or pooled cultures (B). The extracts were tested for MMR activity with 24 fmol of heteroduplex. *These data represent similar results derived from greater than five independent experiments.

5

A. SH clones*

		<u>Repaired substrate (fmol/15 min)</u>	
		3' /CA\	5' G/T
Cell Line			
PMS2-NOT	clone A	10.2	3.5
	clone B	12.7	2.9
	clone C	13.5	5.5
PMS2-WT	clone A	2.8	2.2
	clone B	5.7	4.8
	clone C	4.7	2.9
PMS2-134	clone A	2.5	0.0
	clone B	2.4	0.0
	clone C	5.0	0.5

10

B. Pooled cultures

15

Repaired substrate (fmol/15 min)

20

Cell Line

25

3'G/T 5'G/T 3'/CTG\ 5'/CTG\

30

PMS2-NOT

2.07 +/- 0.09 2.37 +/- 0.37 3.45 +/- 1.35 2.77 +/- 1.37

35

PMS2-WT

1.65 +/- 0.94 1.86 +/- 0.57 1.13 +/- 0.23 1.23 +/- 0.65

40

PMS2-134

0.14 +/- 0.2 0.0 +/- 0.0 1.31 +/- 0.66 0.0 +/- 0.0

Western blots. Equal number of cells were lysed directly in lysis buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1 M 2-mercaptoethanol, 0.001% bromophenol blue) and boiled for 5 minutes. Lysate proteins were separated by electrophoresis on 4-12% Tris-glycine 5 gels (for analysis of full-length hPMS2) or 4-20% Tris-glycine gels (for analysis of hPMS2-134). Gels were electroblotted onto Immobilon-P (Millipore) in 48 mM Tris base, 40 mM glycine, 0.0375% SDS, 20% methanol and blocked overnight at 4°C in Tris-buffered saline plus 0.05% Tween-20 and 5% condensed milk. Filters were probed with a polyclonal 10 antibody generated against residues 2 - 20 of hPMS2 (Santa Cruz Biotechnology, Inc.) and a horseradish peroxidase conjugated goat anti-rabbit secondary antibody, using chemilluminescence for detection (Pierce).

In vitro translation. Linear DNA fragments containing *hPMS2* and *hMLH1* cDNA sequences were prepared by PCR, incorporating sequences 15 for *in vitro* transcription and translation in the sense primer. A full-length *hMLH1* fragment was prepared using the sense primer 5'-ggatcctaatacgactcactatagggaga ccaccatgtcggtggcagg-3' (codons 1-6) and the antisense primer 5'-taagtcttaagtgcattacaac-3' (located in the 3' 20 untranslated region, nt 2411-2433), using a wild-type *hMLH1* cDNA clone as template. A full-length *hPMS2* fragment was prepared with the sense primer 5'-ggatcctaatacgactcactatagggagaccaccatggaacaattgcctgcgg-3' (codons 1- 6) and the antisense primer 5'-aggtagtgaagactctgtc-3' (located in 3' 25 untranslated region, nt 2670-2690) using a cloned *hPMS2* cDNA as template. A fragment encoding the amino-terminal 134 amino acids of hPMS2 was prepared using the same sense primer and the antisense primer 5'-agtcgagttccaaccttcg-3'. A fragment containing codons 135 - 862 of *hPMS135* was generated using the sense primer 30 5'-ggatcctaatacgactcactatagggagaccaccatgtttgatcacaatgg-3' (codons 135-141) and the same antisense primer as that used for the full-length

hPMS2 protein. These fragments were used to produce proteins via the coupled transcription-translation system (Promega). The reactions were supplemented with ^{35}S -labelled methionine or unlabelled methionine, as indicated in the text. The PMS135 and hMLH1 proteins could not be simultaneously radiolabelled and immunoprecipitated because of their similar molecular weights precluded resolution. Lower molecular weight bands are presumed to be degradation products and/or polypeptides translated from alternative internal methionines.

10 Immunoprecipitation. Immunoprecipitations were performed on *in vitro* translated proteins by mixing the translation reactions with 1 µg of the MLH1 specific monoclonal antibody (mAB) MLH14 (Oncogene Science, Inc.), a polyclonal antibody generated to codons 2 - 20 of hPMS2 described above, or a polyclonal antibody generated to codons 843-862 of hPMS2 (Santa Cruz Biotechnology, Inc.) in 400 µl of EBC buffer (50 mM Tris, pH 7.5, 0.1 M NaCl, 0.5% NP40). After incubation for 1 hr at 4°C, protein A **15** sepharose (Sigma) was added to a final concentration of 10% and reactions were incubated at 4°C for 1 hour. Proteins bound to protein A were washed five times in EBC and separated by electrophoresis on 4-20% Tris-glycine gels, which were then dried and autoradiographed.

20 Biochemical assays for mismatch repair. MMR activity in nuclear extracts was performed as described, using 24 fmol of substrate (12,25). Complementation assays were done by adding ~ 100 ng of purified MutL α or MutS α components to 100 μ g of nuclear extract, adjusting the final KCl concentration to 100 mM (4,10,30). The substrates used in these experiments contain a strand break 181 nucleotides 5' or 125 nucleotides 3' to the mismatch. Values represent experiments performed at least in duplicate.
25

EXAMPLE 3: Carboxy Terminus of hPMS2 Mediates Interaction between hPMS2 and hMLH1

To elucidate the mechanism by which hPMS2-134 affected MMR, we analyzed the interaction between hPMS2 and hMLH1. Previous studies have shown that these two proteins dimerize to form a functionally active complex (12, 28). Proteins were synthesized *in vitro* using reticulocyte lysates, employing RNA generated from cloned templates. The full-length hMLH1 and hPMS2 proteins bound to each other and were co-precipitated with antibodies to either protein, as expected (data not shown). To determine the domain of hPMS2 which bound to hMLH1, the amino terminus (codons 1 - 134), containing the most highly conserved domain among mutL proteins (19,24), and the carboxyl terminus (codons 135 - 862) were separately cloned and proteins produced *in vitro* in coupled transcription-translation reactions. When a ³⁵S-labelled, full length hMLH1 protein (Fig. 5A, lane 5) was mixed with the unlabelled carboxyl terminal hPMS2 polypeptide, a monoclonal antibody (mAb) to the carboxyl terminus of hPMS2 efficiently immunoprecipitated the labeled hMLH1 protein (lane 1). No hMLH1 protein was precipitated in the absence of hPMS2 (lane 2). Conversely, when the ³⁵S-labelled carboxyl-terminus of hPMS2 (lane 3) was incubated with unlabelled, full-length hMLH1 protein, an anti-hMLH1 mAb precipitated the hPMS2 polypeptide (lane 4). In the absence of the unlabelled hMLH1 protein, no hPMS2 protein was precipitated by this mAb (lane 6). The same antibody failed to immunoprecipitate the amino-terminus of hPMS2 (amino acids 1-134) when mixed with unlabelled MLH1 protein (Fig. 5B, lane 1). This finding was corroborated by the converse experiment in which radiolabelled hPMS2-134 (Fig. 5C, lane 1) was unable to coprecipitate radiolabelled MLH1 when precipitations were done using an N-terminal hPMS2 antibody (Fig. 5C, lane 2) while this antibody was shown to be able to coprecipitate MLH1 when mixed with wild-type hPMS2 (Fig. 5C, lane 4).

DRAFT DATE 07/26/00

The initial steps of MMR are dependent on two protein complexes, called MutS α and MutL α (14). As the amino terminus of hPMS2 did not mediate binding of hPMS2 to hMLH1, it was of interest to determine whether it might instead mediate the interaction between the MutL α complex (composed of hMLH1 and hPMS2, ref. 12) and the MutS α complex (composed of MSH2 and GTBP, ref. 4). Because previous studies have demonstrated that MSH2 and the MutL α components do not associate in solution (28), we were unable to assay for direct hPMS2-134:MutS α interaction. We therefore used a different approach to address this issue, and attempted to complement nuclear extracts from the various SH cell lines with MutS α or MutL α . If the truncated protein present in the PMS2-134 expressing SH cells was binding to MutS α and lowering its effective concentration in the extract, then adding intact MutS α should rescue the MMR defect in such extracts. Purified MutS α added to such extracts had no effect (Fig. 6). In contrast, addition of intact MutL α to the extract completely restored directional repair to the extracts from PMS2-134 cells (Fig. 6).

The results described above lead to several conclusions. First, expression of the amino-terminus of hPMS2 results in an increase in microsatellite instability, consistent with a replication error (RER) phenotype. That this elevated microsatellite instability is due to MMR deficiency was proven by evaluation of extracts from stably transduced cells. Interestingly, the expression of PMS2-134 resulted in a polar defect in MMR, which was only observed using heteroduplexes designed to test repair from the 5' direction (no significant defect in repair from the 3' direction was observed in the same extracts). Interestingly, cells deficient in hMLH1 also have a polar defect in MMR, but in this case preferentially affecting repair from the 3' direction (5). It is known from previous studies in both prokaryotes and eukaryotes that the separate enzymatic components mediate repair from the two different directions. Our results, in combination with

DRAFT -- PLEASE CITE

those of Drummond *et al.*, strongly suggest a model in which 5' repair is primarily dependent on hPMS2 while 3' repair is primarily dependent on hMLH1. It is easy to envision how the dimeric complex between PMS2 and MLH1 might set up this directionality. The combined results also
5 demonstrate that a defect in directional MMR is sufficient to produce a RER+ phenotype.

We anticipated that the dominant negative function of the PMS2-134 polypeptide resulted from its binding to MLH1 and consequent inhibition of MutL α function. This hypothesis was based in part on the fact that the most highly conserved domain of the *PMS2* gene is located in its amino terminus, and the only known biochemical partner for PMS2 is MLH1. Our binding studies revealed, however, that the carboxyl terminus of PMS2, rather than the highly conserved amino terminus, actually mediated binding to MLH1.
10 This result is consistent with those recently obtained in *S. cerevisiae*, in which the MLH1-interacting domain of PMS1 (the yeast homolog of human PMS2) was localized to its carboxyl-terminus (23). Our add-back experiments additionally showed that the hPMS2-134 mutant was not likely to mediate an interaction with the MutS α complex (Fig. 6). The best
15 explanation at present to explain the various observations made here is that the hPMS2-134 polypeptide does not inhibit the initial steps in MMR, but rather interacts with and inhibits a downstream component of the pathway, perhaps a nuclease required for repair from the 5' direction.
20

The demonstration that the hPMS2-134 mutation can confer a dominant negative MMR defect to transfected cells helps to explain the phenotype of the kindred in which this mutant was discovered. Three individuals from this kindred were found to carry the mutation, a father and his two children. Both children exhibited microsatellite instability in their normal tissues and both developed tumors at an early age, while the father had no evidence of microsatellite instability in his normal cells and was completely healthy at age 35. The only difference known to us with respect to the MMR genes in this family is that the father's mutant allele was
25
30

DEPARTMENT OF BIOLOGY

5

10

expressed at lower levels than the wild-type allele as assessed by sequencing of reverse transcriptase-PCR products of RNA from lymphocytes. The children expressed both alleles at approximately equal levels (Parsons *et al.* and unpublished observations). We suspect that the dominant negative attribute of the *hPMS2-134* mutant will only be manifest when it is present at sufficient concentrations (at least equimolar), thus explaining the absence of MMR deficiency in the father. The reason for the differential expression of the *hPMS2-134* allele in this kindred is not clear, though imprinting is a possibility. Hopefully, the ascertainment of additional, larger kindreds with such mutations will facilitate the investigation of this issue.

References

5 1. Baker S.M., Bronner, C.E., Zhang, L., Plug, A.W., Robatez, M., Warren, G., Elliott, E.A., Yu, J., Ashley, T., Arnheim, N., Bradley, N., Flavell, R.A., and Liskay, R.M. 1995. Male defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis. *Cell* **82**:309-319.

10 2. Bronner, C.E., Baker, S.M., Morrison, P.T., Warren, G., Smith, L.G., Lescoe, M.K., Kane, M., Earabino, C., Lipford, J., Lindblom, A., Tannergard, P., Bollag,R.J., Godwin, A., R., Ward, D.C., Nordenskjold, M., Fishel, R., Kolodner, R., and Liskay, R.M. 1994. Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary non-polyposis colon cancer. *Nature* **368**:258-261.

15 3. de Wind N., Dekker, M., Berns, A., Radman, M., and Riele, H.T. 1995. Inactivation of the mouse *Msh2* gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell* **82**:321-300.

20 4. Drummond, J.T., Li, G.M., Longley, M.J., and Modrich, P. 1995. Isolation of an hMSH2-p160 heterodimer that restores mismatch repair to tumor cells. *Science* **268**:1909-1912.

25 5. Drummond, J.T., Anthoney, A., Brown, R., and Modrich, P. 1996. Cisplatin and adriamycin resistance are associated with MutL α and mismatch repair deficiency in an ovarian tumor cell line. *J.Biol.Chem.* **271**:9645-19648.

30 6. Edelmann, W., Cohen, P.E., Kane, M., Lau, K., Morrow, B., Bennett, S., Umar, A., Kunkel, T., Cattoretti, G., Chagnatti, R., Pollard, J.W., Kolodner, R.D., and Kucherlapati, R. 1996. Meiotic pachytene arrest in MLH1-deficient mice. *Cell* **85**:1125-1134.

7. Fishel, R., Lescoe, M., Rao, M.R.S., Copeland, N.J., Jenkins, N.A., Garber, J., Kane, M., and Kolodner, R. 1993. The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. *Cell* 7:1027-1038.

5

8. Green, S., Issemann, I., and Sheer, E. 1988. A versatile in vivo eucaryotic expression vector for protein engineering. *Nuc. Acid Res.* 16:369.

10

9. Hamilton, S.R., Liu, B., Parsons, R.E., Papadopoulos, N., Jen, J., Powell, S.M., Krush, A.J., Berk, T., Cohen, Z., tetu, B., Kinzler, K.W., and Vogelstein, B. 1995. The molecular basis of Turcot's syndrome. *N. Eng. J. Med.* 332:839-847.

15

10. Holmes, J., Clark, S., and Modrich, P. Strand specific mismatch correction in nuclear extracts of human and *Drosophila melanogaster* cell lines. (1990). *Proc. Natl. Acad. Sci. USA* 87:5837- 5841.

20

11. Leach, F.S., Nicolaides, N.C, Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L.A., Nystrom-Lahti, M., Guan, X.Y., Zhang, J., Meltzer, P.S., Yu, J.W., Kao, F.T., Chen, D.J., Cerosaletti, K.M., Fournier, R.E.K., Todd, S., Lewis, T., Leach R.J., Naylor, S.L., Weissenbach, J., Mecklin, J.P., Jarvinen, J.A., Petersen, G.M., Hamilton, S.R., Green, J., Jass, J., Watson, P., Lynch, H.T., Trent, J.M., de la Chapelle, A., Kinzler, K.W., and Vogelstein, B. 1993. Mutations of a *mutS* homolog in hereditary non-polyposis colorectal cancer. *Cell* 75:1215-1225.

25

12. Li, G.-M., and Modrich, P. 1995. Restoration of mismatch repair to nuclear extracts of H6 colorectal tumor cells by a heterodimer of human *mutL* homologs. *Proc. Natl. Acad. Sci. USA* 92:1950-1954.

30

13. Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N.C., Lynch, H.T., Watson, P., Jass, J.R., Dunlop, M., Wyllie, A., Peltomaki, P., de la Chapelle, A., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. 1996. Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nat. Med.* 2:169-174.

5

14. Modrich, P. 1994. Mismatch repair, genetic stability, and cancer. *Science* 266:1959-1960.

10

15. Nicolaides, N.C., Gualdi, R., Casadevall, C., Manzella, L., and Calabretta, B. 1991. Positive autoregulation of *c-myb* expression via MYB binding in the 5' flanking region of the human *c-myb* gene. *Mol. Cell. Biol.* 11:6166-6176.

15

16. Nicolaides, N.C., Correa, I., Casadevall, C., Travali, S., Soprano, K.J., and Calabretta, B. 1992. The Jun family members , c-JUN and JUND, transactivate the human *c-myb* promoter via an Ap1 like element. *J. Biol. Chem.* 267, 19665-19672.

20

17. Nicolaides, N.C., Papadopoulos, N., Liu, B., Wei, Y.F., Carter, K.C., Ruben, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, C.J., Dunlop, M.G., Hamilton, S.R., Petersen, G.M., de la Chapelle, A., Vogelstein, B., and kinzler, K.W. 1994. Mutations of two PMS homologs in hereditary nonpolyposis colon cancer. *Nature* 371: 75-80.

25

18. Nicolaides N.C., Kinzler, K.W., and Vogelstein, B. 1995. Analysis of the 5' region of PMS2 reveals heterogenous transcripts and a novel overlapping gene. *Genomics* 29:329-334.

30

19. Nicolaides, N.C., Carter, K.C., Shell, B.K., Papadopoulos, N.,
Vogelstein, B., and Kinzler, K.W. 1995. Genomic organization of the
human PMS2 gene family. *Genomics* **30**:195-206.

5 20. Nicolaides, N.C., Palombo, F., Kinzler, K.W., Vogelstein, B., and
Jiricny, J. 1996. Molecular cloning of the N-terminus of GTBP. *Genomics*
31:395-397.

10 21. Palombo, F., Hughes, M., Jiricny, J., Truong, O., Hsuan, J. 1994.
Mismatch repair and cancer. *Nature* **36**:417.

15 22. Palombo, F., Gallinari, P., Iaccarino, I., Lettieri, T., Hughes, M.A.,
Truong, O., Hsuan, J.J., and Jiricny, J. 1995. GTBP, a 160-kilodalton
protein essential for mismatch-binding activity in human cells. *Science*
268:1912-1914.

20 23. Pang, Q., Prolla, T.A., and Liskay, R.M. 1997. Functional domains of
the *Saccharomyces cerevisiae* Mlh1p and Pms1p DNA mismatch repair
proteins and their relevance to human hereditary nonpolyposis colorectal
cancer-associated mutations. *Mol. Cell. Biol.* **17**:4465-4473.

25 24. Papadopoulos, N., Nicolaides, N.C., Wei, Y.F., Carter, K.C., Ruben,
S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M.,
Adams, M.D., Venter, C.J., Dunlop, M.G., Hamilton, S.R., Petersen, G.M.,
de la Chapelle, A., Vogelstein, B., and kinzler, K.W. 1994. Mutation of a
mutL homolog is associated with hereditary colon cancer. *Science*
263:1625-1629.

30 25. Parsons, R., Li, G.M., Longley, M.J., Fang, W.H., Papadopolous, N.,
Jen, J., de la Chapelle, A., Kinzler, K.W., Vogelstein, B., and Modrich, P.

1993. Hypermutability and mismatch repair deficiency in RER⁺ tumor cells.
Cell 75:1227-1236.

5 26. Parsons, R., Li, G.M., Longley, M., Modrich, P., Liu, B., Berk, T.,
Hamilton, S.R., Kinzler, K.W., and Vogelstein, B. 1995. Mismatch repair
deficiency in phenotypically normal human cells. Science 268:738-740.

10 27. Perucho, M. 1996. Cancer of the microsatellite mutator phenotype.
Biol Chem. 377:675-684.

15 28. Prolla, T.A., Pang, Q., Alani, E., Kolodner, R.A., and Liskay, R.M.
1994. MLH1, PMS1, and MSH2 Interaction during the initiation of DNA
mismatch repair in yeast. Science 264:1091-1093.

20 29. Strand, M., Prolla, T.A., Liskay, R.M., and Petes, T.D. Destabilization
of tracts of simple repetitive DNA in yeast by mutations affecting DNA
mismatch repair. 1993. Nature 365:274-276.

30. Su, S.S., Lahue, R.S., Au, K.G., and Modrich, P. 1988. Mispair
specificity of methyl directed DNA mismatch corrections in vitro. J. Biol.
Chem. 263:6829-6835.

SEQUENCE LISTING

5

(1) GENERAL INFORMATION

(i) APPLICANT: NICOLAIDES, NICHOLAS
VOGELSTEIN, BERT
KINZLER, KINZLER

10

(ii) TITLE OF THE INVENTION: A METHOD FOR GENERATING
HYPERMUTABLE ORGANISMS

15

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Banner & Witcoff
- (B) STREET: 1001 G Street, NW
- (C) CITY: Washington
- (D) STATE: DC
- (E) COUNTRY: USA
- (F) ZIP: 20001

20

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ for Windows Version 2.0

25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

30

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:

35

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Kagan, Sarah A
- (B) REGISTRATION NUMBER: 32141
- (C) REFERENCE/DOCKET NUMBER: 01107.73306

40

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 202-508-9100
- (B) TELEFAX: 202-508-9299
- (C) TELEX:

50

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2771 base pairs
- (B) TYPE: nucleic acid

(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 25...2610
(D) OTHER INFORMATION:

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15	CGAGGCAGGAT CGGGTGTTC ATCC ATG GAG CGA GCT GAG AGC TCG AGT ACA Met Glu Arg Ala Glu Ser Ser Ser Thr 1 5	51
20	GAA CCT GCT AAG GCC ATC AAA CCT ATT GAT CGG AAG TCA GTC CAT CAG Glu Pro Ala Lys Ala Ile Lys Pro Ile Asp Arg Lys Ser Val His Gln 10 15 20 25	99
25	ATT TGC TCT GGG CAG GTG GTA CTG AGT CTA AGC ACT GCG GTA AAG GAG Ile Cys Ser Gly Gln Val Val Leu Ser Leu Ser Thr Ala Val Lys Glu 30 35 40	147
30	TTA GTA GAA AAC AGT CTG GAT GCT GGT GCC ACT AAT ATT GAT CTA AAG Leu Val Glu Asn Ser Leu Asp Ala Gly Ala Thr Asn Ile Asp Leu Lys 45 50 55	195
35	CTT AAG GAC TAT GGA GTG GAT CTT ATT GAA GTT TCA GAC AAT GGA TGT Leu Lys Asp Tyr Gly Val Asp Leu Ile Glu Val Ser Asp Asn Gly Cys 60 65 70	243
40	GGG GTA GAA GAA AAC TTC GAA GGC TTA ACT CTG AAA CAT CAC ACA Gly Val Glu Glu Asn Phe Glu Gly Leu Thr Leu Lys His His Thr 75 80 85	291
45	TCT AAG ATT CAA GAG TTT GCC GAC CTA ACT CAG GTT GAA ACT TTT GGC Ser Lys Ile Gln Glu Phe Ala Asp Leu Thr Gln Val Glu Thr Phe Gly 90 95 100 105	339
50	TTT CGG GGG GAA GCT CTG AGC TCA CTT TGT GCA CTG AGC GAT GTC ACC Phe Arg Gly Glu Ala Leu Ser Ser Leu Cys Ala Leu Ser Asp Val Thr 110 115 120	387
55	ATT TCT ACC TGC CAC GCA TCG GCG AAG GTT GGA ACT CGA CTG ATG TTT Ile Ser Thr Cys His Ala Ser Ala Lys Val Gly Thr Arg Leu Met Phe 125 130 135	435
55	GAT CAC AAT GGG AAA ATT ATC CAG AAA ACC CCC TAC CCC CGC CCC AGA Asp His Asn Gly Lys Ile Ile Gln Lys Thr Pro Tyr Pro Arg Pro Arg 140 145 150	483

D955B1473-D9E500

	GGG ACC ACA GTC AGC GTG CAG CAG TTA TTT TCC ACA CTA CCT GTG CGC Gly Thr Thr Val Ser Val Gln Gln Leu Phe Ser Thr Leu Pro Val Arg 155 160 165	531
5	CAT AAG GAA TTT CAA AGG AAT ATT AAG AAG GAG TAT GCC AAA ATG GTC His Lys Glu Phe Gln Arg Asn Ile Lys Lys Glu Tyr Ala Lys Met Val 170 175 180 185	579
10	CAG GTC TTA CAT GCA TAC TGT ATC ATT TCA GCA GGC ATC CGT GTA AGT Gln Val Leu His Ala Tyr Cys Ile Ser Ala Gly Ile Arg Val Ser 190 195 200	627
15	TGC ACC AAT CAG CTT GGA CAA GGA AAA CGA CAG CCT GTG GTA TGC ACA Cys Thr Asn Gln Leu Gly Gln Gly Lys Arg Gln Pro Val Val Cys Thr 205 210 215	675
20	GGT GGA AGC CCC AGC ATA AAG GAA AAT ATC GGC TCT GTG TTT GGG CAG Gly Gly Ser Pro Ser Ile Lys Glu Asn Ile Gly Ser Val Phe Gly Gln 220 225 230	723
25	AAG CAG TTG CAA AGC CTC ATT CCT TTT GTT CAG CTG CCC CCT AGT GAC Lys Gln Leu Gln Ser Leu Ile Pro Phe Val Gln Leu Pro Pro Ser Asp 235 240 245	771
30	TCC GTG TGT GAA GAG TAC GGT TTG AGC TGT TCG GAT GCT CTG CAT AAT Ser Val Cys Glu Glu Tyr Gly Leu Ser Cys Ser Asp Ala Leu His Asn 250 255 260 265	819
35	CTT TTT TAC ATC TCA GGT TTC ATT TCA CAA TGC ACG CAT GGA GTT GGA Leu Phe Tyr Ile Ser Gly Phe Ile Ser Gln Cys Thr His Gly Val Gly 270 275 280	867
40	AGG AGT TCA ACA GAC AGA CAG TTT TTC TTT ATC AAC CGG CGG CCT TGT Arg Ser Ser Thr Asp Arg Gln Phe Phe Ile Asn Arg Arg Pro Cys 285 290 295	915
45	GAC CCA GCA AAG GTC TGC AGA CTC GTG AAT GAG GTC TAC CAC ATG TAT Asp Pro Ala Lys Val Cys Arg Leu Val Asn Glu Val Tyr His Met Tyr 300 305 310	963
50	AAT CGA CAC CAG TAT CCA TTT GTT GTT CTT AAC ATT TCT GTT GAT TCA Asn Arg His Gln Tyr Pro Phe Val Val Leu Asn Ile Ser Val Asp Ser 315 320 325	1011
55	GAA TGC GTT GAT ATC AAT GTT ACT CCA GAT AAA AGG CAA ATT TTG CTA Glu Cys Val Asp Ile Asn Val Thr Pro Asp Lys Arg Gln Ile Leu Leu 330 335 340 345	1059
	CAA GAG GAA AAG CTT TTG TTG GCA GTT TTA AAG ACC TCT TTG ATA GGA Gln Glu Glu Lys Leu Leu Leu Ala Val Leu Lys Thr Ser Leu Ile Gly 350 355 360	1107
	ATG TTT GAT AGT GAT GTC AAC AAG CTA AAT GTC AGT CAG CAG CCA CTG Met Phe Asp Ser Asp Val Asn Lys Leu Asn Val Ser Gln Gln Pro Leu 365 370 375	1155

	CTG GAT GTT GAA GGT AAC TTA ATA AAA ATG CAT GCA GCG GAT TTG GAA Leu Asp Val Glu Gly Asn Leu Ile Lys Met His Ala Ala Asp Leu Glu 380 385 390	1203
5	AAG CCC ATG GTA GAA AAG CAG GAT CAA TCC CCT TCA TTA AGG ACT GGA Lys Pro Met Val Glu Lys Gln Asp Gln Ser Pro Ser Leu Arg Thr Gly 395 400 405	1251
10	GAA GAA AAA AAA GAC GTG TCC ATT TCC AGA CTG CGA GAG GCC TTT TCT Glu Glu Lys Lys Asp Val Ser Ile Ser Arg Leu Arg Glu Ala Phe Ser 410 415 420 425	1299
15	CTT CGT CAC ACA ACA GAG AAC AAG CCT CAC AGC CCA AAG ACT CCA GAA Leu Arg His Thr Thr Glu Asn Lys Pro His Ser Pro Lys Thr Pro Glu 430 435 440	1347
20	CCA AGA AGG AGC CCT CTA GGA CAG AAA AGG GGT ATG CTG TCT TCT AGC Pro Arg Arg Ser Pro Leu Gly Gln Lys Arg Gly Met Leu Ser Ser Ser 445 450 455	1395
25	ACT TCA GGT GCC ATC TCT GAC AAA GGC GTC CTG AGA CCT CAG AAA GAG Thr Ser Gly Ala Ile Ser Asp Lys Gly Val Leu Arg Pro Gln Lys Glu 460 465 470	1443
30	GCA GTG AGT TCC AGT CAC GGA CCC AGT GAC CCT ACG GAC AGA GCG GAG Ala Val Ser Ser His Gly Pro Ser Asp Pro Thr Asp Arg Ala Glu 475 480 485	1491
35	GTG GAG AAG GAC TCG GGG CAC GGC AGC ACT TCC GTG GAT TCT GAG GGG Val Glu Lys Asp Ser Gly His Gly Ser Thr Ser Val Asp Ser Glu Gly 490 495 500 505	1539
40	TTC AGC ATC CCA GAC ACG GGC AGT CAC TGC AGC AGC GAG TAT GCG GCC Phe Ser Ile Pro Asp Thr Gly Ser His Cys Ser Ser Glu Tyr Ala Ala 510 515 520	1587
45	AGC TCC CCA GGG GAC AGG GGC TCG CAG GAA CAT GTG GAC TCT CAG GAG Ser Ser Pro Gly Asp Arg Gly Ser Gln Glu His Val Asp Ser Gln Glu 525 530 535	1635
50	AAA GCG CCT GAA ACT GAC GAC TCT TTT TCA GAT GTG GAC TGC CAT TCA Lys Ala Pro Glu Thr Asp Asp Ser Phe Ser Asp Val Asp Cys His Ser 540 545 550	1683
55	AAC CAG GAA GAT ACC GGA TGT AAA TTT CGA GTT TTG CCT CAG CCA ACT Asn Gln Glu Asp Thr Gly Cys Lys Phe Arg Val Leu Pro Gln Pro Thr 555 560 565	1731
	AAT CTC GCA ACC CCA AAC ACA AAG CGT TTT AAA AAA GAA GAA ATT CTT Asn Leu Ala Thr Pro Asn Thr Lys Arg Phe Lys Lys Glu Glu Ile Leu 570 575 580 585	1779
	TCC AGT TCT GAC ATT TGT CAA AAG TTA GTA AAT ACT CAG GAC ATG TCA Ser Ser Ser Asp Ile Cys Gln Lys Leu Val Asn Thr Gln Asp Met Ser 590 595 600	1827

095534345000

	GCC TCT CAG GTT GAT GTA GCT GTG AAA ATT AAT AAG AAA GTT GTG CCC Ala Ser Gln Val Asp Val Ala Val Lys Ile Asn Lys Lys Val Val Pro 605 610 615	1875
5	CTG GAC TTT TCT ATG AGT TCT TTA GCT AAA CGA ATA AAG CAG TTA CAT Leu Asp Phe Ser Met Ser Ser Leu Ala Lys Arg Ile Lys Gln Leu His 620 625 630	1923
10	CAT GAA GCA CAG CAA AGT GAA GGG GAA CAG AAT TAC AGG AAG TTT AGG His Glu Ala Gln Gln Ser Glu Gly Glu Gln Asn Tyr Arg Lys Phe Arg 635 640 645	1971
15	GCA AAG ATT TGT CCT GGA GAA AAT CAA GCA GCC GAA GAT GAA CTA AGA Ala Lys Ile Cys Pro Gly Glu Asn Gln Ala Ala Glu Asp Glu Leu Arg 650 655 660 665	2019
20	AAA GAG ATA AGT AAA ACG ATG TTT GCA GAA ATG GAA ATC ATT GGT CAG Lys Glu Ile Ser Lys Thr Met Phe Ala Glu Met Glu Ile Ile Gly Gln 670 675 680	2067
25	TTT AAC CTG GGA TTT ATA ATA ACC AAA CTG AAT GAG GAT ATC TTC ATA Phe Asn Leu Gly Phe Ile Ile Thr Lys Leu Asn Glu Asp Ile Phe Ile 685 690 695	2115
30	GTG GAC CAG CAT GCC ACG GAC GAG AAG TAT AAC TTC GAG ATG CTG CAG Val Asp Gln His Ala Thr Asp Glu Lys Tyr Asn Phe Glu Met Leu Gln 700 705 710	2163
35	CAG CAC ACC GTG CTC CAG GGG CAG AGG CTC ATA GCA CCT CAG ACT CTC Gln His Thr Val Leu Gln Gly Gln Arg Leu Ile Ala Pro Gln Thr Leu 715 720 725	2211
40	AAC TTA ACT GCT GTT AAT GAA GCT GTT CTG ATA GAA AAT CTG GAA ATA Asn Leu Thr Ala Val Asn Glu Ala Val Leu Ile Glu Asn Leu Glu Ile 730 735 740 745	2259
45	TTT AGA AAG AAT GGC TTT GAT TTT GTT ATC GAT GAA AAT GCT CCA GTC Phe Arg Lys Asn Gly Phe Asp Phe Val Ile Asp Glu Asn Ala Pro Val 750 755 760	2307
50	ACT GAA AGG GCT AAA CTG ATT TCC TTG CCA ACT AGT AAA AAC TGG ACC Thr Glu Arg Ala Lys Leu Ile Ser Leu Pro Thr Ser Lys Asn Trp Thr 765 770 775	2355
55	TTC GGA CCC CAG GAC GTC GAT GAA CTG ATC TTC ATG CTG AGC GAC AGC Phe Gly Pro Gln Asp Val Asp Glu Leu Ile Phe Met Leu Ser Asp Ser 780 785 790	2403
	CCT GGG GTC ATG TGC CGG CCT TCC CGA GTC AAG CAG ATG TTT GCC TCC Pro Gly Val Met Cys Arg Pro Ser Arg Val Lys Gln Met Phe Ala Ser 795 800 805	2451
	AGA GCC TGC CGG AAG TCG GTG ATG ATT GGG ACT GCT CTT AAC ACA AGC Arg Ala Cys Arg Lys Ser Val Met Ile Gly Thr Ala Leu Asn Thr Ser 810 815 820 825	2499

GAG ATG AAG AAA CTG ATC ACC CAC ATG GGG GAG ATG GAC CAC CCC TGG 2547
Glu Met Lys Lys Leu Ile Thr His Met Gly Glu Met Asp His Pro Trp
830 835 840

5 AAC TGT CCC CAT GGA AGG CCA ACC ATG AGA CAC ATC GCC AAC CTG GGT 2595
Asn Cys Pro His Gly Arg Pro Thr Met Arg His Ile Ala Asn Leu Gly
845 850 855

10 GTC ATT TCT CAG AAC TGACCGTAGT CACTGTATGG AATAATTGGT TTTATCGCAG A 2651
Val Ile Ser Gln Asn
860

15 TTTTTATGTT TTGAAAGACA GAGTCTTCAC TAACCTTTT TGTGTTAAAAA TGAAACCTGC 2711
TACTTAAAAAA AAATACACAT CACACCCATT TAAAAGTGAT CTTGAGAACCC TTTCAAACC 2771

(2) INFORMATION FOR SEQ ID NO:2:

20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 862 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein
(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

30 Met Glu Arg Ala Glu Ser Ser Thr Glu Pro Ala Lys Ala Ile Lys
1 5 10 15
Pro Ile Asp Arg Lys Ser Val His Gln Ile Cys Ser Gly Gln Val Val
20 25 30

35 Leu Ser Leu Ser Thr Ala Val Lys Glu Leu Val Glu Asn Ser Leu Asp
35 40 45
Ala Gly Ala Thr Asn Ile Asp Leu Lys Leu Lys Asp Tyr Gly Val Asp
50 55 60

40 Leu Ile Glu Val Ser Asp Asn Gly Cys Gly Val Glu Glu Asn Phe
65 70 75 80
Glu Gly Leu Thr Leu Lys His His Thr Ser Lys Ile Gln Glu Phe Ala
85 90 95

45 Asp Leu Thr Gln Val Glu Thr Phe Gly Phe Arg Gly Glu Ala Leu Ser
100 105 110
Ser Leu Cys Ala Leu Ser Asp Val Thr Ile Ser Thr Cys His Ala Ser
115 120 125
Ala Lys Val Gly Thr Arg Leu Met Phe Asp His Asn Gly Lys Ile Ile
130 135 140
Gln Lys Thr Pro Tyr Pro Arg Pro Arg Gly Thr Thr Val Ser Val Gln
145 150 155 160

50 Gln Leu Phe Ser Thr Leu Pro Val Arg His Lys Glu Phe Gln Arg Asn
165 170 175
Ile Lys Lys Glu Tyr Ala Lys Met Val Gln Val Leu His Ala Tyr Cys
180 185 190
Ile Ile Ser Ala Gly Ile Arg Val Ser Cys Thr Asn Gln Leu Gly Gln

	195	200	205
	Gly Lys Arg Gln Pro Val Val Cys Thr Gly	Gly Ser Pro Ser Ile Lys	
5	210 215	220	
	Glu Asn Ile Gly Ser Val Phe Gly Gln Lys	Gln Leu Gln Ser Leu Ile	
	225 230	235 240	
	Pro Phe Val Gln Leu Pro Pro Ser Asp Ser Val Cys Glu Glu Tyr Gly		
	245 250	255	
	Leu Ser Cys Ser Asp Ala Leu His Asn Leu Phe Tyr Ile Ser Gly Phe		
	260 265	270	
10	Ile Ser Gln Cys Thr His Gly Val Gly Arg Ser Ser Thr Asp Arg Gln		
	275 280	285	
	Phe Phe Phe Ile Asn Arg Arg Pro Cys Asp Pro Ala Lys Val Cys Arg		
	290 295	300	
	Leu Val Asn Glu Val Tyr His Met Tyr Asn Arg His Gln Tyr Pro Phe		
15	305 310	315 320	
	Val Val Leu Asn Ile Ser Val Asp Ser Glu Cys Val Asp Ile Asn Val		
	325 330	335	
	Thr Pro Asp Lys Arg Gln Ile Leu Leu Gln Glu Glu Lys Leu Leu Leu		
	340 345	350	
20	Ala Val Leu Lys Thr Ser Leu Ile Gly Met Phe Asp Ser Asp Val Asn		
	355 360	365	
	Lys Leu Asn Val Ser Gln Gln Pro Leu Leu Asp Val Glu Gly Asn Leu		
	370 375	380	
	Ile Lys Met His Ala Ala Asp Leu Glu Lys Pro Met Val Glu Lys Gln		
25	385 390	395 400	
	Asp Gln Ser Pro Ser Leu Arg Thr Gly Glu Glu Lys Lys Asp Val Ser		
	405 410	415	
	Ile Ser Arg Leu Arg Glu Ala Phe Ser Leu Arg His Thr Thr Glu Asn		
	420 425	430	
30	Lys Pro His Ser Pro Lys Thr Pro Glu Pro Arg Arg Ser Pro Leu Gly		
	435 440	445	
	Gln Lys Arg Gly Met Leu Ser Ser Ser Thr Ser Gly Ala Ile Ser Asp		
	450 455	460	
	Lys Gly Val Leu Arg Pro Gln Lys Glu Ala Val Ser Ser Ser His Gly		
35	465 470	475 480	
	Pro Ser Asp Pro Thr Asp Arg Ala Glu Val Glu Lys Asp Ser Gly His		
	485 490	495	
	Gly Ser Thr Ser Val Asp Ser Glu Gly Phe Ser Ile Pro Asp Thr Gly		
	500 505	510	
40	Ser His Cys Ser Ser Glu Tyr Ala Ala Ser Ser Pro Gly Asp Arg Gly		
	515 520	525	
	Ser Gln Glu His Val Asp Ser Gln Glu Lys Ala Pro Glu Thr Asp Asp		
	530 535	540	
	Ser Phe Ser Asp Val Asp Cys His Ser Asn Gln Glu Asp Thr Gly Cys		
45	545 550	555 560	
	Lys Phe Arg Val Leu Pro Gln Pro Thr Asn Leu Ala Thr Pro Asn Thr		
	565 570	575	
	Lys Arg Phe Lys Lys Glu Ile Leu Ser Ser Ser Asp Ile Cys Gln		
	580 585	590	
50	Lys Leu Val Asn Thr Gln Asp Met Ser Ala Ser Gln Val Asp Val Ala		
	595 600	605	
	Val Lys Ile Asn Lys Lys Val Val Pro Leu Asp Phe Ser Met Ser Ser		
	610 615	620	
	Leu Ala Lys Arg Ile Lys Gln Leu His His Glu Ala Gln Gln Ser Glu		
55	625 630	635 640	

Gly Glu Gln Asn Tyr Arg Lys Phe Arg Ala Lys Ile Cys Pro Gly Glu
645 650 655
Asn Gln Ala Ala Glu Asp Glu Leu Arg Lys Glu Ile Ser Lys Thr Met
660 665 670
5 Phe Ala Glu Met Glu Ile Ile Gly Gln Phe Asn Leu Gly Phe Ile Ile
675 680 685
Thr Lys Leu Asn Glu Asp Ile Phe Ile Val Asp Gln His Ala Thr Asp
690 695 700
Glu Lys Tyr Asn Phe Glu Met Leu Gln Gln His Thr Val Leu Gln Gly
705 710 715 720
10 Gln Arg Leu Ile Ala Pro Gln Thr Leu Asn Leu Thr Ala Val Asn Glu
725 730 735
Ala Val Leu Ile Glu Asn Leu Glu Ile Phe Arg Lys Asn Gly Phe Asp
740 745 750
15 Phe Val Ile Asp Glu Asn Ala Pro Val Thr Glu Arg Ala Lys Leu Ile
755 760 765
Ser Leu Pro Thr Ser Lys Asn Trp Thr Phe Gly Pro Gln Asp Val Asp
770 775 780
Glu Leu Ile Phe Met Leu Ser Asp Ser Pro Gly Val Met Cys Arg Pro
785 790 795 800
20 Ser Arg Val Lys Gln Met Phe Ala Ser Arg Ala Cys Arg Lys Ser Val
805 810 815
Met Ile Gly Thr Ala Leu Asn Thr Ser Glu Met Lys Lys Leu Ile Thr
820 825 830
25 His Met Gly Glu Met Asp His Pro Trp Asn Cys Pro His Gly Arg Pro
835 840 845
Thr Met Arg His Ile Ala Asn Leu Gly Val Ile Ser Gln Asn
850 855 860

DRAFT - DRAFT

WE CLAIM:

1. A method for making a hypermutable cell, comprising the step of:
introducing into a mammalian cell a polynucleotide comprising a
dominant negative allele of a mismatch repair gene, whereby the cell
becomes hypermutable.
5
2. The method of claim 1 wherein the polynucleotide is introduced by
transfection of a suspension of cells *in vitro*.
3. The method of claim 1 wherein the mismatch repair gene is *PMS2*.
4. The method of claim 1 wherein the mismatch repair gene is human *PMS2*.
10
5. The method of claim 1 wherein the mismatch repair gene is human
MLH1.
6. The method of claim 1 wherein the mismatch repair gene is human *PMS1*.
7. The method of claim 1 wherein the mismatch repair gene is human
MSH2.
15
8. The method of claim 4 wherein the allele comprises a truncation
mutation.
9. The method of claim 4 wherein the allele comprises a truncation mutation
at codon 134 as shown in SEQ ID NO: 1.
10. The method of claim 9 wherein the truncation mutation is a thymidine at
nucleotide 424 of wild-type *PMS2* as shown in SEQ ID NO: 1.
20
11. The method of claim 1 wherein the polynucleotide is introduced into a
fertilized egg of an animal.
12. The method of claim 11 wherein the fertilized egg is subsequently
implanted into a pseudo-pregnant female whereby the fertilized egg develops
into a mature transgenic animal.
25
13. The method of claim 12 wherein the mismatch repair gene is *PMS2*.
14. The method of claim 12 wherein the mismatch repair gene is human
PMS2.
15. The method of claim 12 wherein the mismatch repair gene is human
MLH1.
30

09559499-0726.00

16. The method of claim 12 wherein the mismatch repair gene is human
PMS1.

17. The method of claim 12 wherein the mismatch repair gene is human
MSH2.

5 18. The method of claim 14 wherein the allele comprises a truncation
mutation.

19. The method of claim 14 wherein the allele comprises a truncation
mutation at codon 134 as shown in SEQ ID NO: 1.

10 20. The method of claim 19 wherein the truncation mutation is a thymidine
at nucleotide 424 of wild-type *PMS2* as shown in SEQ ID NO: 1.

21. A homogeneous composition of cultured, hypermutable, mammalian
cells which comprise a dominant negative allele of a mismatch repair gene.

22. The isolated hypermutable cell of claim 21 wherein the mismatch repair
gene is *PMS2*.

15 23. The isolated hypermutable cell of claim 21 wherein the mismatch repair
gene is human *PMS2*.

24. The isolated hypermutable cell of claim 21 wherein the mismatch repair
gene is human *MLH1*.

25. The isolated hypermutable cell of claim 21 wherein the mismatch repair
gene is human *PMS1*.

20 26. The isolated hypermutable cell of claim 21 wherein the mismatch repair
gene is human *MSH2*.

27. The isolated hypermutable cell of claim 21 wherein the cells express a
protein consisting of the first 133 amino acids of hPMS2.

25 28. A hypermutable transgenic mammal wherein at least 50% of the cells of
the mammal comprise a dominant negative allele of a mismatch repair gene.

29. The hypermutable transgenic animal of claim 28 comprising a protein
which consists of the first 133 amino acids of human PMS2.

30 30. A method for generating a mutation in a gene of interest comprising the
steps of:

growing a mammalian cell comprising the gene of interest and a dominant negative allele of a mismatch repair gene, wherein the cell is hypermutable;

5 testing the cell to determine whether the gene of interest harbors a mutation.

31. The method of claim 30 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.

32. The method of claim 30 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.

10 33. The method of claim 30 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

34. The method of claim 30 wherein the step of testing comprises analyzing the phenotype of the gene of interest.

15 35. The method of claim 30 wherein the mammalian cell is made by the process of introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into a mammalian cell, whereby the cell becomes hypermutable.

36. The method of claim 35 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.

20 37. The method of claim 35 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.

38. The method of claim 35 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

25 39. The method of claim 35 wherein the step of testing comprises analyzing the phenotype of the gene of interest.

40. A method for generating a mutation in a gene of interest comprising the steps of:

30 growing a mammal comprising the gene of interest and a polynucleotide encoding a dominant negative allele of a mismatch repair gene;

SEARCHED - INDEXED - DRAFTED - SERIALIZED

testing the mammal to determine whether the gene of interest harbors a mutation.

41. The method of claim 40 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.
5
42. The method of claim 40 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.
43. The method of claim 40 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.
44. The method of claim 40 wherein the step of testing comprises analyzing the phenotype of the gene of interest.
10
45. The method of claim 40 wherein the mammal is made by the process of introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into a mammal, whereby the mammal becomes hypermutable.
15
46. The method of claim 45 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.
47. The method of claim 45 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.
48. The method of claim 45 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.
20
49. The method of claim 45 wherein the step of testing comprises analyzing the phenotype of the gene of interest.
50. A hypermutable transgenic mammal made by the method of claim 45.
25
51. The transgenic mammal of claim 50 wherein the mammal is a primate.
52. The transgenic mammal of claim 50 wherein the mismatch repair gene is *PMS2*.
53. The transgenic mammal of claim 50 wherein the mismatch repair gene is human *PMS2*.
30
54. The transgenic mammal of claim 50 wherein the mismatch repair gene is human *MLH1*.

55. The transgenic mammal of claim 50 wherein the mismatch repair gene is human *PMS1*.

56. The transgenic mammal of claim 50 wherein the mismatch repair gene is human *MSH2*.

57. The transgenic mammal of claim 50 wherein the allele comprises a truncation mutation.

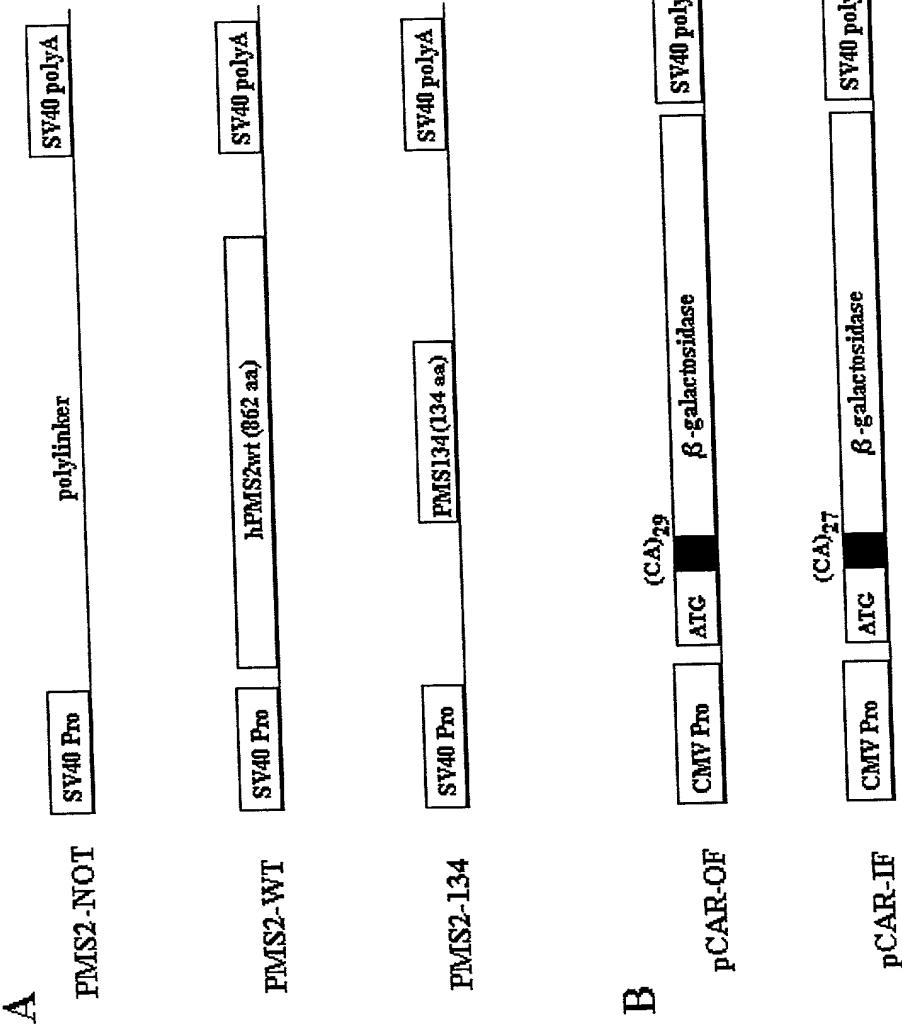
58. The transgenic mammal of claim 50 wherein the allele comprises a truncation mutation at codon 134 as shown in SEQ ID NO: 1.

10 59. The transgenic mammal of claim 58 wherein the truncation mutation is a thymidine at nucleotide 424 of wild-type *PMS2* as shown in SEQ ID NO: 1.

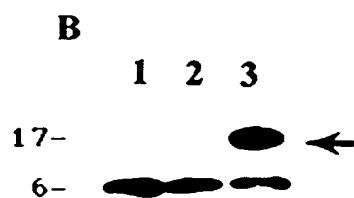
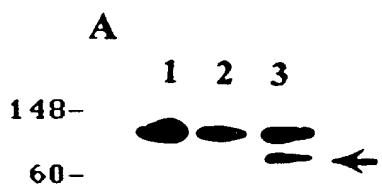
A METHOD FOR GENERATING HYPERMUTABLE ORGANISMS

ABSTRACT

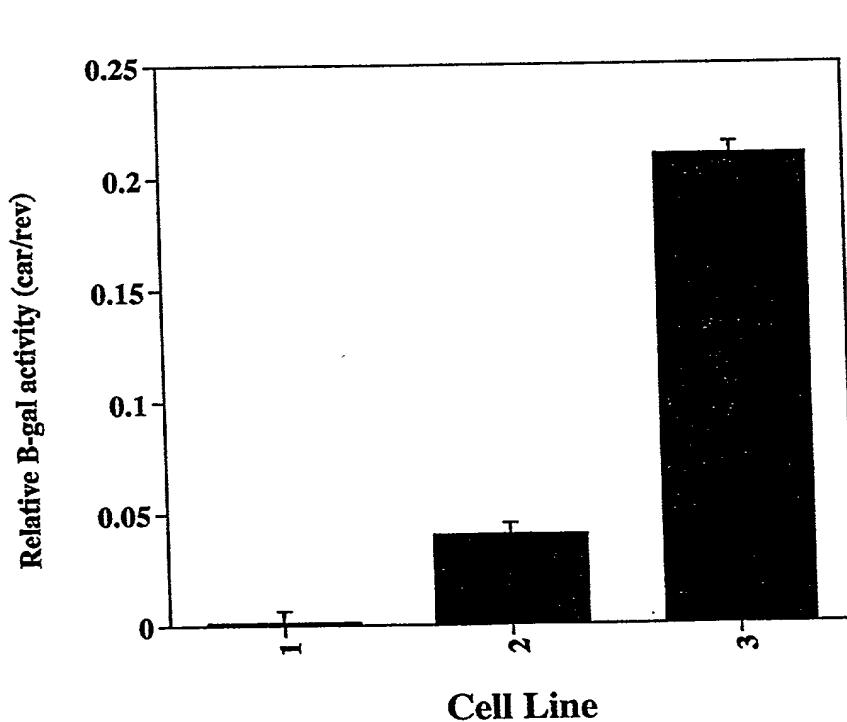
5 Dominant negative alleles of human mismatch repair genes can be used to generate hypermutable cells and organisms. By introducing these genes into cells and transgenic animals, new cell lines and animal varieties with novel and useful properties can be prepared more efficiently than by relying on the natural rate of mutation.



Nicolaides et.al.
Figure 2



C



00326554 "S-4" 5-6-60

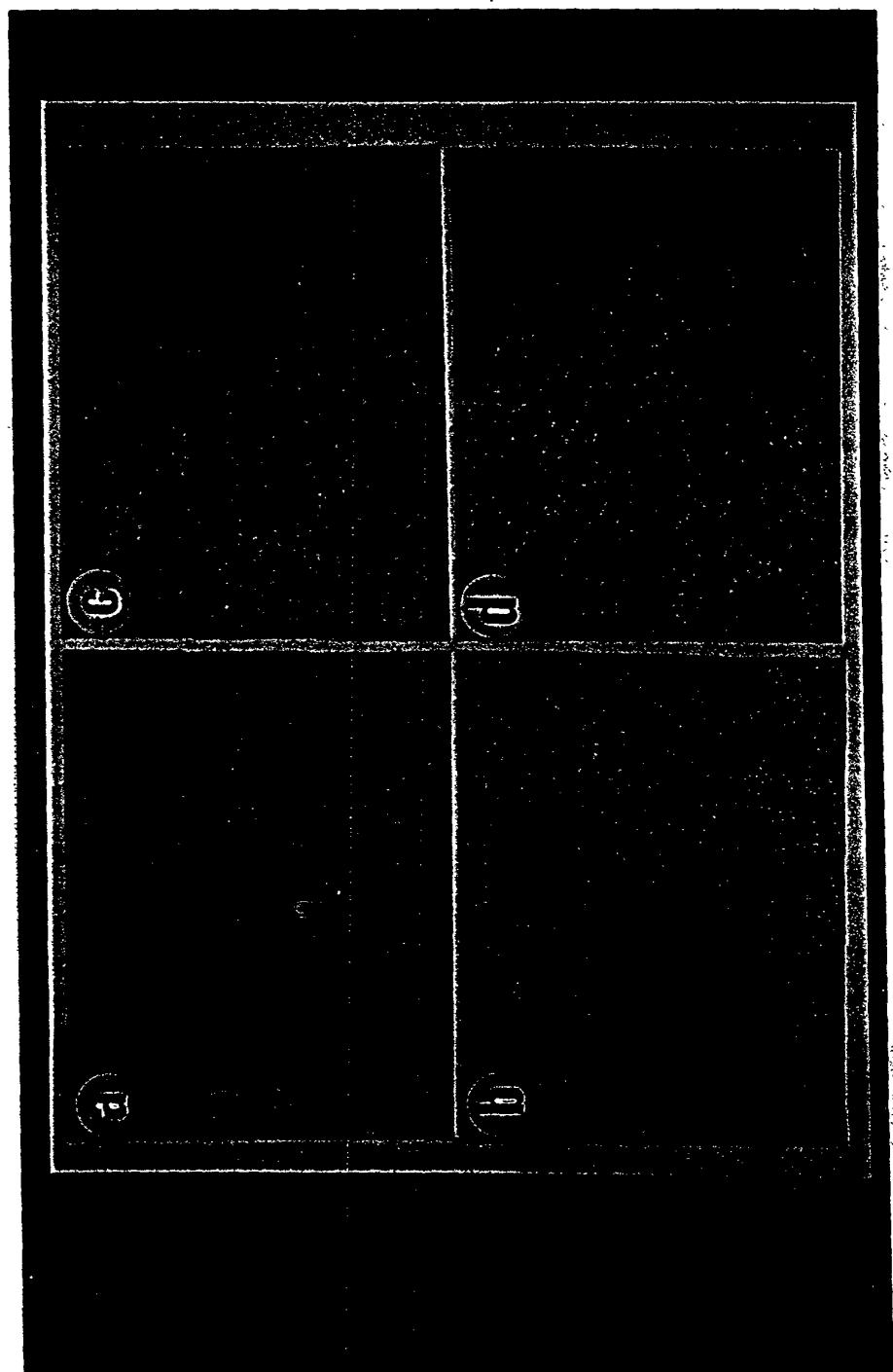


Fig 3

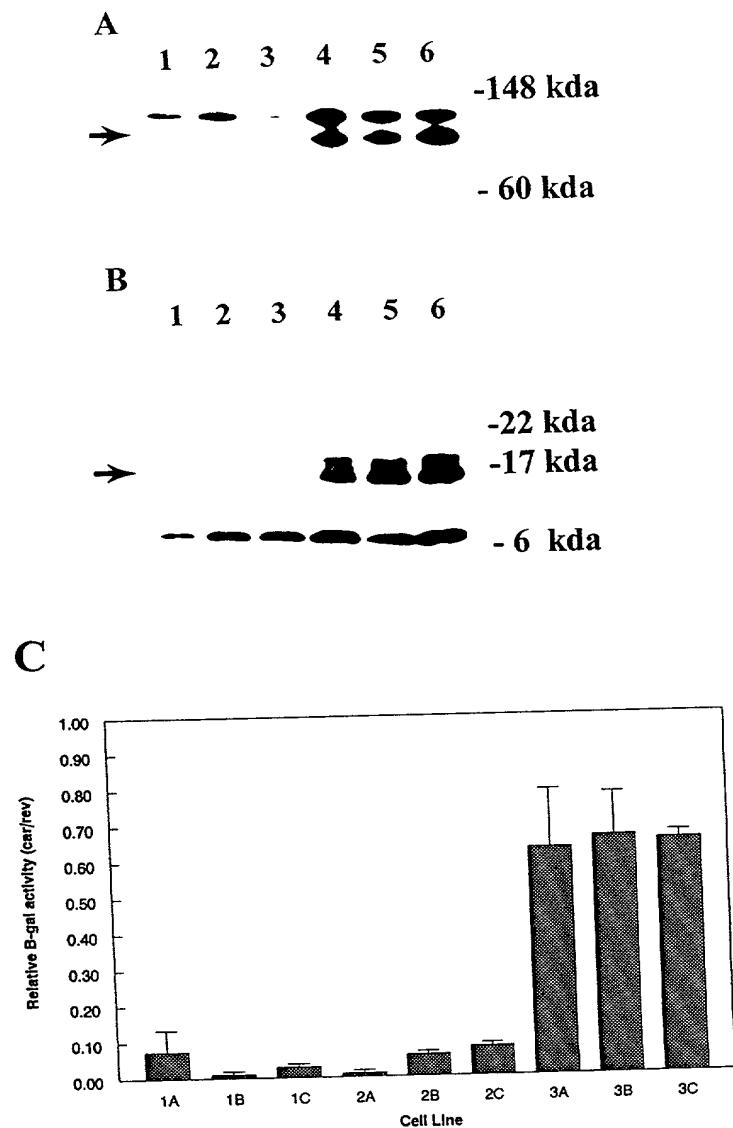


Fig 4

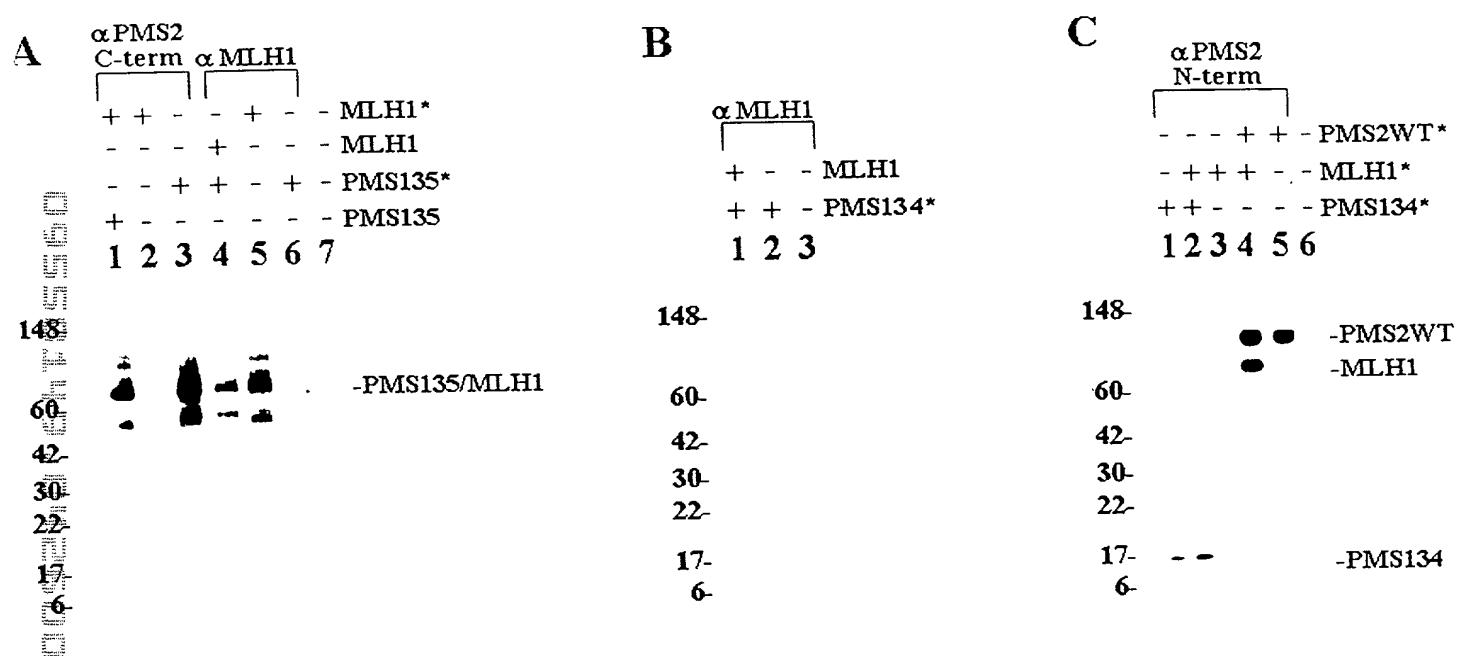


Fig 5

